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BIOTECHNOLOGY METHODS

Selective enrichment and production of highly urease active bacteria by non-sterile (open) chemostat culture

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Abstract In general, bioprocesses can be subdivided into naturally occurring processes, not requiring sterility (e.g., beer brewing, wine making, lactic acid fermentation, or biogas digestion) and other processes (e.g., the production of enzymes and antibiotics) that typically require a high level of sterility to avoid contaminant microbes overgrowing the production strain. The current paper describes the sustainable, non-sterile production of an industrial enzyme using activated sludge as inoculum. By using selective conditions (high pH, high ammonia concentration, and presence of urea) for the target bacterium, highly active ureolytic bacteria, physiologically resembling *Sporosarcina pasteurii* were reproducibly enriched and then continuously produced via chemostat operation of the bioreactor. When using a pH of 10 and about 0.2 M urea in a yeast extract-based medium, ureolytic bacteria developed under aerobic chemostat operation at hydraulic retention times of about 10 h with urease levels of about 60 μmol min^{-1} ml^{-1} culture. For cost minimization at an industrial scale the costly protein-rich yeast extract medium could be replaced by commercial milk powder or by lysed activated sludge. Glutamate, molasses, or glucose-based media did not result in the enrichment of ureolytic bacteria by the chemostat. The concentration of intracellular urease was sufficiently high such that the produced raw effluent from the reactor could be used directly for biocementation in the field.

Keywords Chemostat · Urease · Non-sterile · Activated sludge · Biocementation · Soil stabilization

Introduction

Urease (urea amidohydrolase, EC 3.5.1.15) is a nickel-containing enzyme that catalyzes the hydrolysis of urea to carbon dioxide and ammonia. It is synthesized by a wide range of bacteria, fungi, several species of yeast, and some plants [5, 16]. The urease enzyme has been well studied from a clinical perspective as it is related to the virulence of pathogenic bacteria [11, 22, 27, 44].

In recent years, microbial urease has received increased interest as a catalyst for $CaCO_3$ precipitation in the presence of Ca^{2+} and urea. This process is termed microbially induced carbonate precipitation (MICP) and has been proposed as a natural method for various applications such as protection of decayed ornamental stone [21], improvement of the durability of cementitious materials [12, 33], and biocementation [9, 10].

The process of biocementation for the purpose of soil strengthening has been the subject of research [2, 44] and has been submitted as a patent application [20]. Pilot scale trials on a 100 m^3 scale and field trials have been carried out with this technology [40, 43]. However, prior to full-scale usage on projects such as soil liquefaction or embankment stabilization, an economically viable production of the ureolytic bacteria is necessary. The production of urease-positive bacteria on a larger scale would become prohibitively expensive if traditional biotechnology processes relying on full process sterility were used, as about 5 m^3 of bacterial culture has been found to be necessary for 100 m^3 of sand to be cemented [39]. Until now,

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to obtain a reliable and constantly high urease activity, this large demand of urease is only achieved by cultivating urease-positive cultures under sterile conditions [13, 45]. Even slight contamination of the yeast extract-based medium will result in contamination and the growth of urease-negative bacteria and process failure [44].

The production of urease-positive bacteria for the purpose of in situ biocementation or other MICP applications represents a major cost factor. This may limit the commercialization of the technology. The key reason for the high cost is the labor, energy, equipment, and transport costs involved when producing the bacteria by biotechnology, and the cost of growth media sterilization is in the order of US$0.46–0.66 per liter [44]. Thus, the ability to cultivate the organism with a high level of urease activity, under non-sterile conditions is highly desirable for cost minimization and on-site production with simple technology.

Urease activity is widely distributed in soil and aquatic environments, where it plays an essential role in nitrogen metabolism [7]. The enrichment of urease-positive strains from the environment has been studied. Fujita et al. [14] reported that all groundwater samples collected during the study tested positive for urease activity. Al-Thawadi and Cord-Ruwisch [3] successfully enriched and isolated several ureolytic strains from soil and secondary activated sludge by providing selective conditions (high pH, presence of urea up to 5 M). Varenyam et al. [42] isolated two types of urease-positive bacterial strains from highly alkaline cement samples.

As ureolytic bacteria can be enriched from the environment under selective conditions, it is fair to assume that non-sterile cultivation of urease-positive microorganisms should be feasible. Urease activity of *Sporosarcina pasteurii* was not impaired by up to 50 % (w/w) of contaminants after 48 h of incubation [44]. However, the long-term cultivation of ureolytic bacteria under selective and non-sterile conditions has not been established.

The aim of this study is to make use of the natural capacity of the highly urease active alkaliphiles of the *S. pasteurii* type to thrive in conditions that are toxic to most other bacteria, namely the presence of high concentrations of free ammonia (NH_3), for the development of a production method for bacterial urease and to test whether continuous production in a non-sterile chemostat is feasible.

Materials and methods

Batch enrichments of urease-positive microorganisms

For the batch enrichment of urease-positive bacteria, 10 ml of raw activated sludge (5 g/l, dry weight, collected from Woodman Point wastewater treatment plant, Western Australia) together with 90 ml of different growth media was placed in a series of shaking flasks (250 ml). 20 g/l yeast extract (YE) (Analytical Reagent (AR) grade, Becton-Dickinson) and 0.1 mM $NiCl_2$ (AR grade, Chem-Supply) were added to all enrichments; however, the concentrations of ammonium sulfate (AR grade, Chem-Supply) and urea (AR grade, Chem-Supply) varied in each enrichment according to the following: enrichment 1 and 2, 0.17 M ammonium sulfate; enrichment 3, 0.17 M urea; and enrichment 4, 0.17 M ammonium sulfate and 0.17 M urea. The pH of the enrichment was monitored and controlled at a desired set point by automatically dosing HCl (2 M) or NaOH (10 M). The initial pH of all enrichments was 9.5 and controlled constantly except for enrichment 1, where it was allowed to drift to about 8. All enrichments were tested in duplicate.

All flasks were incubated in a water bath at 28 ± 1 °C for 36 h with shaking speed of 180 rpm. The pH monitoring system consisted of National Instruments Labview (Version 7.2) software and Labjack (U12, Labjack Corporation) data acquisition card.

Chemostat cultivation

Operation system

The chemostat consisted of a 1-l stirred glass bioreactor with 0.5 l operating volume. It was continuously fed with fresh medium from a 2-l feed vessel. A predetermined hydraulic retention time (HRT) of around 10 h was established by using a calibrated peristaltic influent pump with a speed of 50 ml/h. The intake of the decanting pump, which was set to pump faster than the influent pump, was placed at the water level of 0.5 l keeping the operating volume constant. The stirring speed was set at 400 rpm and an air pump (1 l/min) was connected to the reactor delivering a specific mass transfer coefficient (k_La) of about 60 h^{-1}. The air pump was controlled by simple on–off control resulting in an oxygen steady state concentration between 5 and 6 mg/l throughout the experiment as monitored by a polarographic oxygen electrode (Mettler-Toledo, Ltd). The pH of the reactor was also controlled to the set point as described above. During the cultivation, the culture temperature was kept at 28 ± 1 °C. The culture effluent was tested regularly for urease activity and biomass concentration.

Operation procedures of chemostat with YE-based growth medium

Initially, the chemostat reactor was operated under batch mode by cultivating 500 ml of growth medium with 10 % (v/v) activated sludge, 20 g/l YE, 0.17 M ammonium

sulfate, and 0.17 M urea with the pH controlled at 9.5. When the urease activity of the batch enrichment had reached more than 5 μmol min^{-1} ml^{-1} after about 24 h of growth, a transition from batch to chemostat cultivation was initiated.

After shifting to chemostat cultivation by continuously feeding medium containing 20 g/l YE and 0.17 M urea, three chemostat reactors were separately tested at pH 9.5, 9.75, and 10, to evaluate the effect of pH on urease activity development.

In order to evaluate the effect of concentration of YE and acetate on urease activity development, the concentrations of YE (0–40 g/l) and sodium acetate (0–40 g/l) were changed gradually when the chemostat culture reached steady state under each condition. Throughout these tests, the YE-based growth media contained 0.1 mM of $NiCl_2$ and 0.17 M urea at a constant pH of 10.

Operation procedures of chemostat with non-YE-based growth medium

As an alternative to the YE-based medium, several other organic substrates (g/l), including milk powder (40), activated sludge (8), monosodium glutamate (MSG) (10), sugar cane molasses (20), and glucose were tested (20) (Table 1) by just substituting the YE with the alternative organic substrate while keeping other components in the medium constant. All the chemostat cultivation in this experiment was kept at constant pH 10 and a feeding rate of 50 ml/h. For each organic substrate tested, the chemostat was started from a mixture of 90 % (v/v) of the new test growth medium with 10 % (v/v) of the chemostat culture (as inoculum) that had established on YE-based medium as specified in the "Results" section. Then the chemostat reactor was operated for about 10 HRT by continuously feeding with the new test medium.

It should be noted that the concentrated activated sludge (8 g/l, dry weight) provided as feed largely comprised intact cells, which were inaccessible as a feed supply to the enriched culture. Attempts to lyse the bacterial cells were made by exposing them to high alkalinity (pH 12.5) and high ammonium (ammonium sulfate 85 mM) for 24 h. Then, as described above, the chemostat was started from a mixture containing the lysed activated sludge (450 ml) and the chemostat culture (50 ml, served as inoculum, established on YE-based medium). During the chemostat cultivation, the lysed activated sludge consisting of the cell extract, high pH, and high ammonia was continuously fed into the reactor at a constant rate of 50 ml/h. As the lysed activated sludge already had a high pH, less additional NaOH solution was required for the maintenance of reactor pH at constant level of 10.

Table 1 Organic substrates for chemostat cultivation of urease-positive microorganisms under non-sterile conditions

Organic substrates	Characteristics	Protein content
Yeast extract	Water-soluble fraction of autolysed yeast cells	66 % [44]
Sodium acetate	Simple organic substrate as carbon and energy source	0 %
Milk powder	Partially soluble	25 % minimum
Activated sludge	Concentrated (8 g/l), non-lysed bacterial biomass	55 % [44]
Sodium glutamate	Monosodium salt of glutamic acid	0 %
Glucose	Simple sugar	0 %
Molasses	Inexpensive carbohydrate by-product from sugar (sugarcane) production	0 %

Monitoring methods

Biomass measurement (OD_{600})

Biomass concentration was recorded as dry weight per volume. Because of the good correlation between biomass concentration and optical density (Eq. 1), routine biomass monitoring was carried out by optical density measurements using a spectrophotometer (600 nm). All samples were diluted to a range of 0.2–1 of absorbance prior to measuring.

A correlation of biomass concentration (dry weight) and optical density was established and expressed as the following equation (Eq. 1):

$$C\ (\text{biomass concentration},\ \text{g/l}) = 0.44 \times \text{OD}\ (600\ \text{nm}) \quad (R^2 = 0.998) \qquad (1)$$

Urease activity

The urease activity of the culture can be determined from the rate of change of conductivity as it causes a conversion of non-ionic substrates (urea) to ionic products (NH_4^+ and CO_3^{2-}) [45]. Thus 1 ml of the culture was added to 5 ml of 3 M urea and 4 ml of deionized water and the relative conductivity change was recorded over 5 min at 25 °C. According to Whiffin [44], 1 mS cm^{-1} min^{-1} corresponds to 11.11 μmol min^{-1} ml^{-1} of urease activity. Considering the dilution of the culture during the activity measurement by a factor of 10, the urease activity of the tested culture is 111.1 μmol min^{-1} ml^{-1}.

The extracellular urease was determined by taking 1 ml of the supernatant from the centrifuged culture (15,000 rpm for 5 min) and measuring the urease activity as described previously. Specific urease activity of the

bacteria was defined as the urease activity (culture urease activity minus supernatant urease activity) per milligram of biomass. All samples were adjusted to pH 9 by adding 1 M HCl prior to the activity measurement.

Results

Effect of enrichments on urease activity produced from activated sludge

A series of batch enrichments (Fig. 1) was set up to enrich urease active bacteria with activated sludge as the inoculum (10 %). High concentrations of ammonium or the ammonium precursor urea and a high pH were used in the batch enrichments to favor the development of alkaliphilic ureolytic bacteria. The pH and urea and ammonia concentrations of the batch enrichments were set as shown in Fig. 1.

After 36 h of incubation in the presence of ammonium sulfate at pH 9.5 the activated sludge had produced 4 μmol min^{-1} ml^{-1} of urease activity (Fig. 1, enrichment 2), while no significant activity was obtained in the absence of ammonium sulfate, even when urea was present instead. This suggests that the ammonium sulfate was crucial for heightening the growth of ureolytic bacteria and suppressing the urease-negative bacteria. Furthermore, the highest urease activity (11 μmol min^{-1} ml^{-1}) was achieved with urea addition in the presence of ammonium (Fig. 1, enrichment 4). There was no detectable urease activity in the enrichment with uncontrolled pH (Fig. 1, enrichment 1), where the pH decreased from 9.5 to 8 throughout the batch cultivation.

Continuously producing urease activity

Instead of batch cultivation, chemostat cultivation can enable the continued selection of the most adapted organisms; further it enables constant and well-controlled process conditions such as pH and ammonia levels.

The transition from batch to chemostat operation with the same growth medium at controlled pH of 9.5 did not maintain the urease activity level that had developed during the batch growth. Instead, the urease activity continuously declined, while the biomass continuously increased to 7.5 (OD_{600}) (Fig. 2a). This obvious overgrowing of urease active bacteria by non-urease active contaminants is consistent with the observation that the urease active *Proteus vulgaris* was overgrown under non-sterilized chemostat cultivation [44]. Creating more severe and potentially more selective conditions by increasing the pH set point from 9.5 to 9.75 prolonged the steady state of urease activity under chemostat operation for up to 20 h. However, a similar tendency of decrease in urease activity was observed after this 20-h steady state (Fig. 2b).

Operation at pH 10 resulted in an initial drop in overall biomass level and urease activity (Fig. 2c). However, after continued operation for a further two cycles of HRT, the total and specific urease activity increased markedly. Continued chemostat operation at pH 10 resulted in sustained high urease activity levels (>10 μmol min^{-1} ml^{-1}) for more than 140 h (>14 HRT). The results of the chemostat experiments show that a highly urease active bacteria could be selectively enriched and sustainably grown by maintaining the pH at 10 in the presence of 340 mM of NH_4^+/NH_3 (produced from 170 mM urea addition) (Fig. 2).

Effect of YE and sodium acetate concentration on chemostat urease production

When eliminating the costly YE component from the medium, the previously enriched ureolytic bacteria were no longer able to grow (Table 2). The biomass concentration and urease activity increased with the concentration of YE present, indicating that YE had been growth-limiting in this culture.

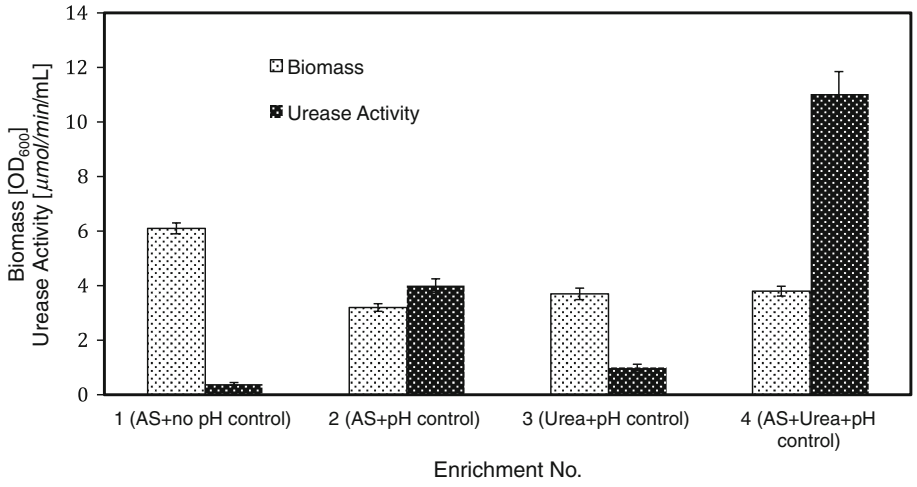

Fig. 1 Effect of ingredients of enrichments on urease activity and biomass production after 36 h of cultivation. These enrichments were inoculated with 10 % (v/v) of activated sludge and incubated for 36 h at 28 °C; 0.17 M of urea and/or ammonium sulfate (AS) was added where indicated

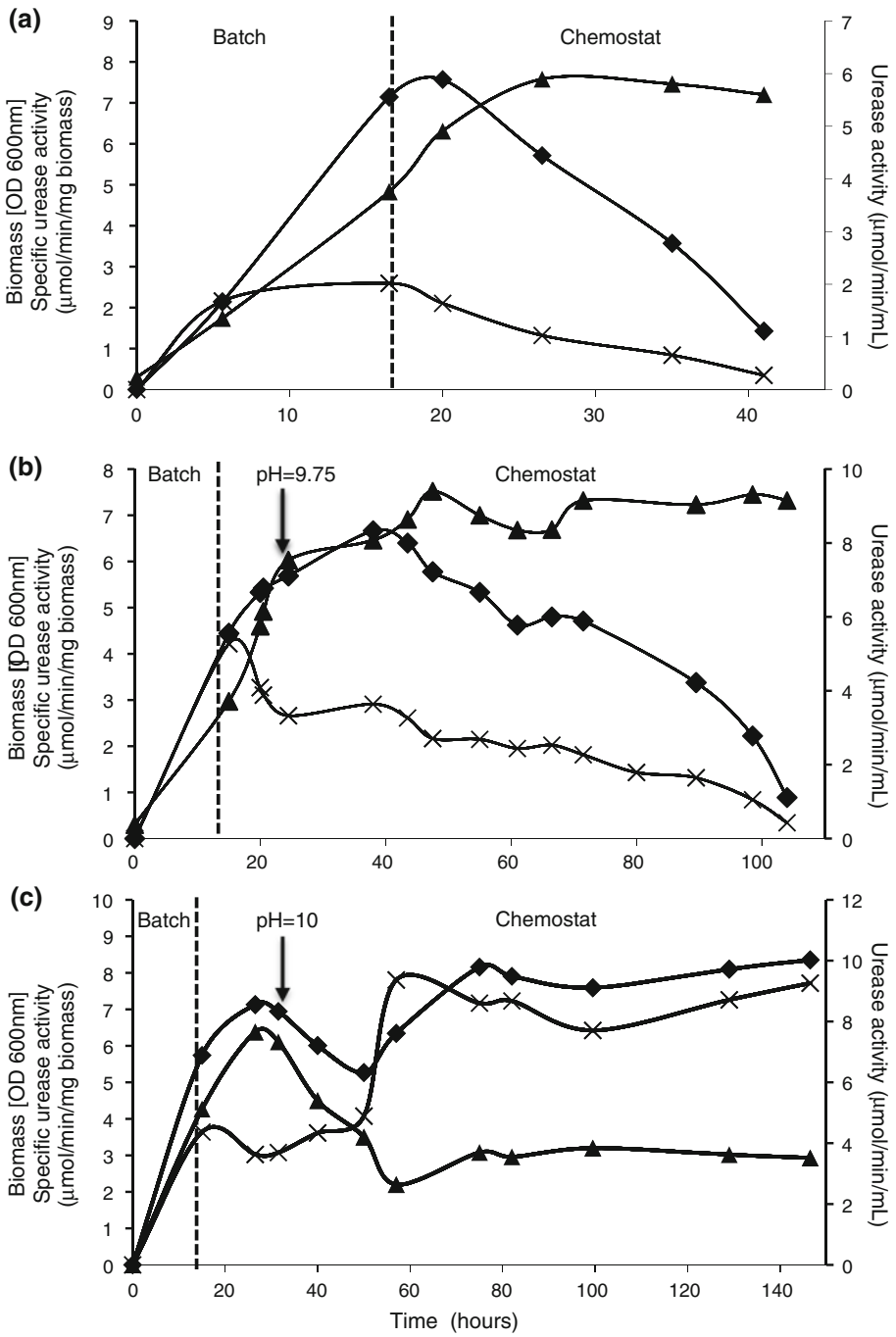

Fig. 2 Transition from batch enrichment (at pH 9.5) to continuous chemostat cultivation (HRT 10 h) of ureolytic bacteria with 10 % of activated sludge as the inoculum. Urease activity (*filled diamonds*), specific urease activity (*cross symbols*), and biomass (*filled triangles*). *Broken line* indicates the transition from batch to chemostat and *arrows* indicate change of pH set point by the pH controller

Whiffin [44] reported that the addition of acetate (sodium acetate) enhanced the total urease activity of *S. pasteurii* by promoting more bacterial growth while keeping the specific activity at a similar level. In our experiments the presence of acetate (up to 20 g/l) did not enhance growth but increased the specific urease activity of the culture (Table 2). This phenomenon was observed both in batch (data not shown) and chemostat cultures (Table 2) indicating that the responsible microorganism in the chemostat enrichment culture might be different from the strain of *S. pasteurii.* By combining the optimum YE and acetate concentrations a urease activity of 58 µmol $\cdot$ min^{-1} ml^{-1} was obtained with the chemostat culture described. This is about three times higher than reported from pure culture experiments with *S. pasteurii* [2, 44].

Economic organic substrates for growth of ureolytic bacteria

For large-scale and economic cultivation of bacteria, complex media are used and often consist of waste or by-products from the food or agricultural industries [8, 32].

Table 2 Summary of the urease activity yield with different organic sources under chemostat cultivation

Substrates (g/l)		Biomass (g/l)	Standard deviation	UA (U/ml)	Standard deviation	SUA (U/mg biomass)	Yield efficiency (U/g organic substrates
YE medium							
YE (0)	SA (40)	0.13	0.01	1.6	0.08	12.1	40
YE (5)	SA (40)	0.87	0.09	12.4	0.56	14.2	275
YE (10)	SA (40)	1.14	0.06	15.5	0.35	13.6	310
YE (20)	SA (40)	1.42	0.06	19.1	0.83	13.4	320
YE (40)	SA (0)	3.90	0.12	26.3	1.68	6.8	657
YE (40)	SA (10)	3.90	0.13	49.3	0.42	12.6	986
YE (40)	SA (20)	3.77	0.26	58.2	0.95	15.5	970
YE (40)	SA (40)	3.72	0.20	58.1	2.24	15.7	726
Alternative organic medium							
Milk powder (40)	SA (10)	N/A	N/A	16.7	0.22	N/A	280
Activated sludge (8)		N/A	N/A	8.8	0.67	N/A	267
MSG (10)		0.14	0.01	0.39	0.01	2.8	13
Glucose (20)		0.09	0.01	0.5	0.01	1.1	13
Molasses (20)		N/A	N/A	0	0	N/A	0

The average values of urease activity (UA) and biomass ($n = 3$) were presented when the chemostat cultures with different organic sources reached steady state. 0.17 M urea was present in all media and the pH of the culture was maintained at 10

YE yeast extract, *SA* sodium acetate, *UA* urease activity (average urease activity at steady state), *SUA* specific urease activity, *N/A* not available

Several alternative organic substrates, including inexpensive protein and carbohydrate sources, were investigated to replace laboratory grade YE (Table 2). The effect of alternative substrates was tested by operating the chemostat continuously for at least ten cycles of HRT using the previously urease active bacteria enriched on YE-based medium (40 g/l YE and 20 g/l acetate, Table 2) as inoculum (10 %).

Two of the alternative economic feed sources (milk powder and hydrolyzed activated sludge) largely comprise proteins and are hence a potentially suitable substrate for YE-degrading bacteria. Milk powder, a partly water-soluble substrate, supported the growth of urease active bacteria but only led to urease activities of about 16.7 μmol min^{-1} ml^{-1}, about three times less than the activity obtained in YE medium (Table 2).

In principle, the lowest cost source of protein hydrolysate could be derived from waste bacterial protein such as activated sludge. Here, we hydrolyzed activated sludge by using an alkaline NH_3 solution (see "Materials and methods") as this pretreatment is compatible with the subsequent use in the urease-producing chemostat culture (also high pH and NH_3 levels). The produced activated sludge extract sustained the production of urease activity by non-sterile chemostat cultivation comparable to that of milk powder (Table 2).

The trials of testing other substrates indicated that neither glutamic acid (added as MSG), the principal component (more than 9 %) of YE, nor sugar cane molasses or glucose addition supported the development of ureolytic bacteria (Table 2).

Significance of pH 10 for stable chemostat urease production

A significant decrease in biomass and specific urease activity was observed when the pH of the mature culture [started from the culture established on YE-based medium (40 g/l YE, 20 g/l acetate, 0.17 M urea, and pH 10, Table 2) as inoculum (10 %)] decreased to 9.5 (Fig. 3). This indicated that maintaining the pH at 10 was essential for maintaining a population with high urease activity. The increased supernatant urease activity at lower pH (Fig. 3, until 80 h) also revealed that urease enzyme had been released from the cells, which is likely due to the bacterial cells lysis. Increasing the pH to 9.8 did not recover the declined urease.

Only when the pH of the chemostat was increased back to 10 did the urease activity recover to 60 μmol min^{-1} ml^{-1} (Fig. 3), showing that the subtle pH change between 9.5 and 10 was critical for selective urease production by chemostat enrichments. Longer periods of operation in excess of 10 days showed that as long as the pH was maintained at about 10, the chemostat consistently produced high levels of urease activity (data not shown).

Role of urea in sustainable urease production by chemostat culture

To determine the need for urea, the urea concentration of the medium fed to the chemostat [started from the culture established on YE-based medium (40 g/l YE, 20 g/l acetate, 0.17 M urea, and pH 10, Table 2) as inoculum

(10 %)] was halved and then doubled (Fig. 4). The specific urease activity in the chemostat culture was clearly stimulated by increased urea concentrations (Fig. 4). This suggests that the urease enzyme was significantly induced by the presence of urea, similar to that by *Proteus* and *Providencia* species [28, 34]. However, with increasing urea concentration a decrease in biomass was also observed (Fig. 4).

After replacing the urea by two alternate ammonium sources (ammonium sulfate and ammonium hydroxide) the urease activity and biomass decreased immediately by more than 80 % within about five cycles of HRT (Fig. A1). However, the activity of the culture recovered after subjecting it to urea again.

Specific urease activity and bacterial types observed

The level of urease activity produced from the non-sterile chemostat operation described here compares well with the activities described for pure cultures of ureolytic bacteria and other urease sources (Fig. 5). Microscopic investigation of the chemostat enrichments showed rod-shaped spore-forming bacteria of a length of about 2 µm which is consistent with the description of *S. pasteurii*, isolated by Al-Thawadi and Cord-Ruwisch [3] from selective batch enrichments. The isolation of the pure culture and its characterization and identification are in progress but are beyond the scope of this article.

Reproducibility of sustained chemostat steady states

The results above show that after establishing a batch enrichment culture of ureolytic bacteria, the culture could be specifically controlled and operated for prolonged non-sterile continuous chemostat operation. This technique could be quite useful for the economic production of urease for industrial applications such as biocementation [4, 9, 10, 40, 41], as it avoids the need for sterility. Before it can be used in industry, the reproducibility of reaching and maintaining high levels of urease activity after the transition from batch enrichment to continuous chemostat operation must be demonstrated.

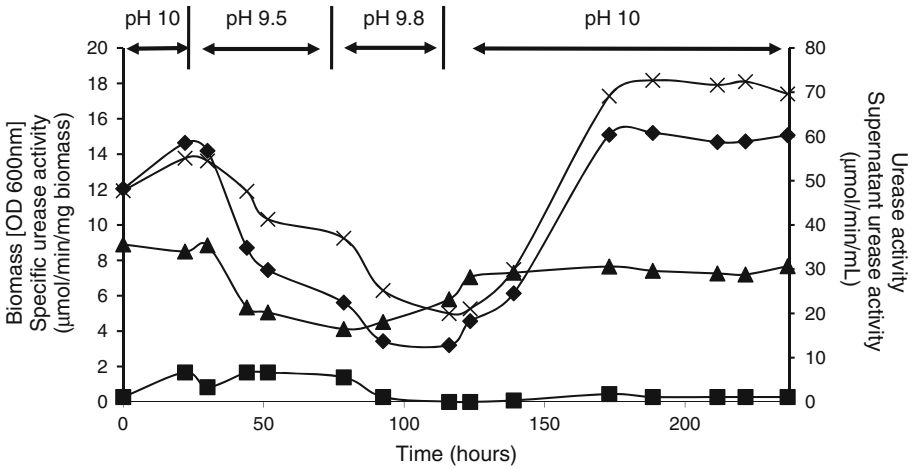

Fig. 3 Effect of pH on biomass (*filled triangles*), urease activity (*filled diamonds*), specific urease activity (*cross symbols*), and supernatant urease activity (*filled squares*) of the culture at chemostat cultivation. Before this trial the chemostat had been operated for more than 100 h at pH 10 with continuous feed containing 0.17 M urea, 40 g/l of YE, and 20 g/l of sodium acetate

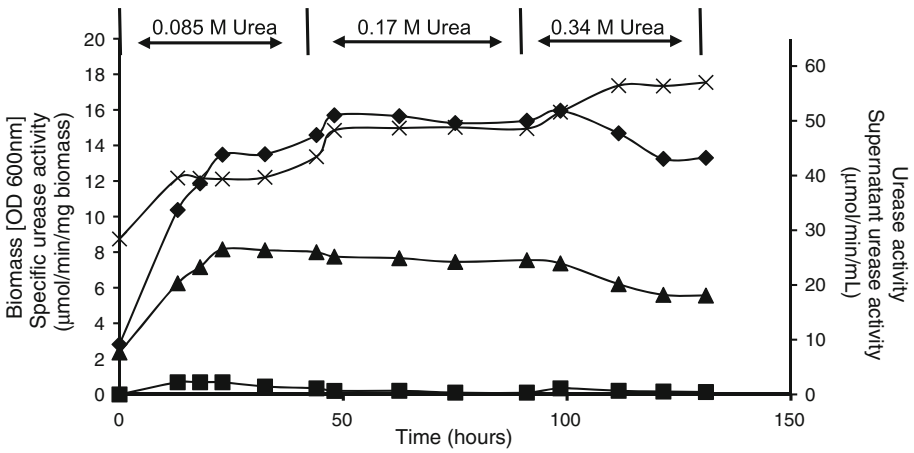

Fig. 4 Effect of urea concentration on biomass (*filled triangles*), urease activity (*filled diamonds*), specific urease activity (*cross symbols*), and supernatant urease activity (*filled squares*) of the chemostat enrichment culture with constant pH of 10; 40 g/l of YE and 20 g/l of sodium acetate were continuously supplied at a hydraulic retention time of 10 h

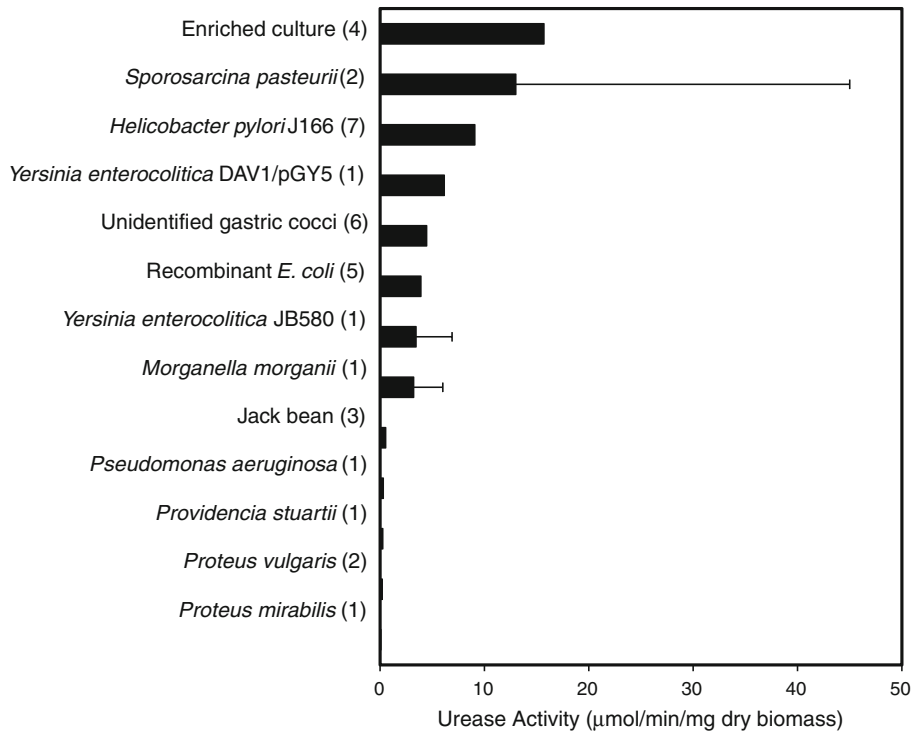

Fig. 5 Comparison of urease activities of currently enriched culture with that of other urease-positive organisms (after Whiffin [44]). *Bars* indicate the urease activity under normal conditions of growth. *Error bars* indicate the reported variable range of urease activity. The reported urease activities for organisms were converted from activity per milligram of protein to activity per milligram of dry cell weight according to the general protein content of prokaryotic organisms of 55 % [24]. Specific urease activity of jack bean was reported as urease activity per milligram of jack bean. Activities were sourced from (1) [46]; (2) [44]; (3) [43]; (4) the present study; (5) [30]; (6) [22]; (7) [15]

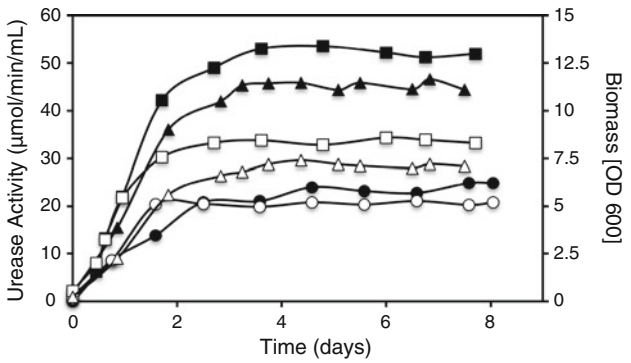

Fig. 6 Development of sustainable steady state conditions of urease activity (*filled symbols*) and biomass level (*open symbols*) during the selective enrichment of ureolytic bacteria under non-sterile chemostat operation. Medium composition (g/l) YE and acetate respectively was *squares* 40 and 20, *triangles* 40 and 20, *spheres* 20 and 10

The reproducibility of the technique described has been tested by restarting three independent chemostat cultivations with different batches of activated sludge as the sole inoculum (Fig. 6) and operating under chemostat conditions for at least 7 days at HRT of 10 h. In all three trials of chemostat operation (in the presence of 0.17 M urea at constant pH of 10) more than 20 µmol min^{-1} ml^{-1} of urease activity was reproducibly produced. This level of urease activity is sufficient for the direct application in biocementation, which requires at least 10 µmol min^{-1} ml^{-1} of urease activity [44].

Discussion

Selective cultivation of ureolytic bacteria

This work demonstrated an example bioprocess that leads to the production of a specific enzyme by using an open process under continuous non-sterile operation. Non-sterile growth and maintenance of bacteria has been widely used in industry processes, such as wastewater treatment, bio-fuel production, biogas production, etc., where specific bacteria are selectively grown by feeding with particular substrates. Recently the production of high levels of polyhydroxyalkanoates (PHAs) by continuous bacterial enrichment cultures has been described [19]. The term eco-biotechnology has been used for such processes where instead of expensive high-tech sterile bio-processing, the knowledge of the physiology of the target organisms is used to streamline process conditions for its selective continued cultivation [38]. The non-sterile chemostat production of enzymes, however, is not a common process, with perhaps the production of crude cellulose being an exception [23].

In this study, a method is described that allowed the production of the enzyme urease by ureolytic bacteria from enriched activated sludge under non-sterile conditions. The selective conditions for the enrichment of the urease-positive bacteria included a high pH of 10, and high concentrations of urea as substrate and its hydrolysis product ammonia, to control the excessive growth of urease-negative microorganisms.

Role of ammonia in specific production of ureolytic bacteria

Although a nitrogen source for most bacteria, ammonia is described to be toxic to bacteria when present in high concentration owing to its well-known cytotoxic effect [17, 29]. While high concentrations of ammonia are detrimental to most cells, it may be of advantage to specific ureolytic bacteria, such as *S. pasteurii*, assisting in their ATP generation. The ATP generation in the presence of high concentrations of free NH_3 has been discussed as follows. NH_3 molecules of the bulk solution migrate through the cells membrane via passive diffusion, and speciate into NH_4^+ ions owing to the moderate cytoplasmic pH [6, 31]. Then the efflux of intracellular cations (here, NH_4^+) other than H^+ can result in an increased membrane potential, $\Delta\Psi$, which drives the influx of protons into the cells, resulting in ATP generation [18]. Therefore, the presence of high concentrations of NH_3 could be a factor leading to the selective enrichment of ureolytic bacteria while inhibiting urease-negative strains.

Role of urea in the selective enrichment of ureolytic bacteria

It is also interesting to note that under chemostat conditions the current enriched ureolytic bacteria will only grow and produce the urease enzyme in the presence of urea. This suggests that the strains enriched here were dependent on the presence of urea for both the production of urease activity and also for cell growth. Several *S. pasteurii* isolates are believed to be able of producing ATP through urea hydrolysis [26].

Required nutrients for non-sterile continuous production of ureolytic bacteria

The cost of laboratory grade media is prohibitively expensive for large-scale cultivations of microorganisms. Inexpensive substrates such as Vegemite (paste of yeast extract) and corn steep liquor have been used for the *S. pasteurii* cultivation [1, 44]. The nutritional profiles for the ureolytic bacteria enriched in this study indicated a need for protein-based media. The milk powder and lysed activated sludge could significantly reduce the cost of urease and may be considered as potential economical industrial substrates for large-scale production of urease activity.

Alkaline extraction has been shown to be a useful method to prepare wet activated sludge as a substrate for bioprocesses [25, 35, 36]. However such processes require additional chemicals. In this study the additional costs normally arising from chemical hydrolysis could be partly avoided by using chemicals, namely ammonia and sodium hydroxide, that are required anyway for effective urease production.

Industrial applications

The current trials demonstrate the reproducibility of the technique that allows a continuous on-site production of urease from local bacteria with low technology. This technology can be up-scaled to enable large-scale production of urease similar to other non-sterile processes such as anaerobic digestion, wine making, and activated sludge processes. This would be useful for large-scale applications of urease in the field, such as biocementation, where approximately 1,000 m^3 of gravel soils has been stabilized by microbially induced calcium carbonate precipitation via ureolytic bacteria [37].

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